PRIMARY RESEARCH PAPER | *Philippine Journal of Systematic Biology*
DOI 10.26757/pjsb2020a14003

Metagenomic approach for the isolation and cultivation of cellulose-degrading myxobacteria

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Abstract

Cellulose-degrading myxobacteria are common soil microorganisms with unique morphologies and are united forming the distinct *Sorangium* and *Byssovorax* clades in the *Polyangiaceae* family, order *Myxococcales*. Cells of these fascinating bacteria can glide, form fruiting bodies and degrade agar and cellulose. However, phenotypic studies based on the swarm, fruiting bodies and cellulose degradation pattern have shown many dissimilarities among strains suggesting more potential to identify new species in *Sorangium* clade. Currently, nine validly described species are recognized capable of decomposing cellulose in myxobacteria, of which eight species belong to the genus *Sorangium* and one species to *Byssovorax*. With the increasing number of species recently validated in *Sorangium*, likely there will also be more species of *Byssovorax* that can be found in the environment. BLAST search analysis of *Byssovorax* 16S rRNA gene sequences in NCBI public database (GenBank) revealed 97-98.8 % similarity with sequences derived from clones of uncultured bacteria. In *Sorangium,* six 16S rRNA gene sequences derived from clones of uncultured bacteria were identified occupying novel branches and exhibiting 96.9% - 98.6% similarities with type strains. Molecular phylogenetic analysis confirmed the affiliations of these clones within *Sorangium* and *Byssovorax* clades, indicating potentially new species within these genera. Surprisingly, one sequence derived from a clone (DEN_SIP_103) may potentially represent a novel genus only if this strain can be successfully cultured in the future.

Keywords: myxobacteria, metagenomics, 16S rRNA gene, *Sorangium, Byssovorax,* cellulose-degradation

Introduction

The Myxobacteria (order *Myxococcales*) are regarded as one of the most highly evolved Gram-negative prokaryotes in the *Delta Proteobacteria* capable of producing complex fruiting bodies under nutrient-depriving condition. Many myxobacteria prey on microorganisms, which are used as a nutrient source for growth and multicellular development while some possess lytic enzymes for macromolecule degradation of cellulose, chitin, and agar. Due to the slow growth and difficulties in cultivation, very few laboratories around the world focus on exploring the diversity of myxobacteria. Although these bacteria are distributed worldwide in different environments, the soil appears to be the most common source for isolation. The tropical climate is considered abundant for myxobacteria and this is reflected in the species diversity in this region (Dawid, 2000).

The search for myxobacteria in the past decades was driven mainly by biotechnological applications for finding novel antibiotics and anti-cancer drugs (Shimkets *et al.,* 2006; Reichenbach & Dworkin, 1992), although it is not limited to these applications. Myxobacteria are also known for the production of steroids (Kohl *et al.,* 1983; Zeggel, 1993; Gawas *et al.,* 2011), diverse polyunsaturated fatty acids (Garcia *et al.,* 2011; Gemperlein *et al.,* 2014) and compounds with anti-viral (Plaza *et al.,* 2012) and insecticidal activity (Kunze *et al.,* 1993). The discovery of novel species, genera, and families in the last years has widened the understanding of myxobacterial biology, systematics and secondary metabolites (Garcia *et al.,* 2010). Some of these myxobacterial isolates are regarded as facultative anaerobe (Sanford *et al.,* 2002), microaerophile (Garcia *et al.,* 2009; 2016), microaerotolerant (Garcia *et al.,* 2018), obligate

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Date Submitted: 24 August 2019
Date Accepted: 19 May 2020

halophile (Iizuka *et al.,* 1998; 2003a-b; Fudou *et al.,* 2002; Ojika *et al.,* 2008), and halotolerant (Iizuka *et al.,* 2013).

To date, all validly described cellulose-degrading myxobacterial species are phylogenetically clustered in the genus *Sorangium* and *Byssovorax*. Between these two genera, *Byssovorax* seems rarely isolated and it appears with only one species and two strains are identified (Reichenbach 2005; Reichenbach *et al.,* 2006). *Byssovorax* also seems to be a rare but interesting source of novel bioactive compounds in myxobacteria. So far, two novel natural compound families have been isolated in this genus and these include the antifungal and cytotoxic cruentaren (Kunze *et al.,* 2006 - 2007), and the cytotoxic and antiviral macrolide haprolid (Steinmetz *et al.,* 2016; Pietschmann *et al.,* 2012). On the other hand, *Sorangium* is much established and can be considered a major producer of secondary metabolites among myxobacteria. The anticancer compound epothilone appears to be the most useful myxobacterial compound from *Sorangium*; it is became marketed by Bristol-Myers Squibb for the treatment of metastatic breast cancer (Mulzer, 2009).

Since cellulose-degrading myxobacteria are industrially valuable due to their medical applications, further studies to discover new species within producer genera and related taxa are highly motivated. To uncover potential new *Sorangium* species and determine the rarity of *Byssovorax* in the environment, BLAST search from the metagenomes in public databases (GenBank) was performed using the type species 16S rRNA gene sequences.

Isolation of cellulolytic myxobacteria and their phenotypic variations

Cellulose-degrading myxobacteria are common organisms in soils with decaying plants. Isolation is based on baiting with filter paper on mineral salt agar (e.g. MS21, a modification of Stan21). MS21 contains (g per liter, w/v), KNO_3 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCl_2 \cdot 2H_2O$ 0.5, $FeCl_3$, 0.02, $MnSO_4 \cdot 7H_2O$ 0.1, HEPES 2.38, trace element solution (Drews, 1974) 100 mL/L, yeast extract 0.02, Bacto agar 10, pH adjusted to 7.0 with KOH; supplemented with K_2HPO_4 0.4 after autoclaving (Garcia & Müller, 2014). Soil materials are placed on the edge of a filter paper and the plate culture is usually incubated for more than two weeks. They can also be isolated in ST21 agar containing KNO_3 and filter paper (Reichenbach *et al.,* 2005). Plain water agar baited with *Escherichia coli* is also used as a method for the isolation of *Byssovorax cruenta*; however, this appears doubtful due to a contrary statement that no growth was exhibited on this medium even if baited with filter paper (Reichenbach *et al.,* 2006).

The cellulose-degrading myxobacteria can be recognized by the appearance of swarming and fruiting bodies on filter paper (Fig. 1a-1c). The swarm colony of *Sorangium* and *Byssovorax* can be identified by their cigar-shaped, non-flexuous, non-motile and phase-dark rods (Fig. 1d). Purification is commonly centered on fruiting bodies and swarming colony on mineral salts agar and Baker's yeast medium (e.g. VY/2 agar). Farthest and cleanest swarm edges are cut out while fruiting bodies can be clean by serially washing in drops of sterile water to decrease the adhering microbial contaminants. Unlike many myxobacteria, cellulose-degrading strains are much slow-growing and easily get contaminated, thus the use of antibiotics is sometimes necessary for the purification steps (Reichenbach, 1983; Reichenbach *et al.,* 2006).

Cellulolytic myxobacteria are remarkable for their colors and patterns of filter paper decomposition (Fig. 2a-2d). Unlike *Byssovorax*, *Sorangium* exhibits variations in cellulose decomposition including shrinking of the filter paper (Fig. 2a), decomposition of cellulose fibers (Fig. 2b-2c) and to complete disappearance, and with of only fruiting bodies left (Fig. 2d). In mineral salts agar with filter paper strips, *Byssovorax* produces a hole in the paper as it swarms (Garcia & Müller, 2014). *Byssovorax* also differs from *Sorangium* for its soft and veiny swarm which appears bloody red on filter paper and agar (Fig 3a -3d). In some myxobacteria, the colors are associated with carotenoids (Jansen *et al.,* 1995; Reichenbach & Kleinig, 1971) and probably with their variants.

Sorangium produces different morphology patterns of sporangioles (Fig. 4a - 4d) and swarms (Fig. 5a-5d). Some strains show differences in color and sizes of sporangioles which could be arranged in chains (Fig. 4a), clusters (Fig. 4b-4d) or sometimes solitary. Some sporangioles are clustered compactly or loosely arrange in sori. The most common sporangiole color appears to be orange; black is quite rare (Fig. 4c). In general, *Sorangium* sporangioles are smaller compared with most myxobacteria. In *S. cellulosum* DSM 14627^T, sporangioles measure 20-30µm in diameter (Garcia & Müller, 2014) while those in other strains of the same species are much bigger (e.g. 40-80µm). In contrast, *Byssovorax* sporangioles are three to four times larger (80-140µm diameter) than *Sorangium.*

The swarming pattern and morphology of *Sorangium* also vary. Swarms on agar may spread fast or slow, appear as nets (Fig. 5a), thick and branching veins (Fig. 5b) or as curtains or circular bands as in some members of family *Polyangiaceae* (Fig. 5c). Other strains form a tough and stringy swarm that often breaks, rips or cut the agar, and crumples the filter paper (Fig. 5d). In few strains, the swarm may appear deeply depressed in the agar as in some *Nannocystis* species (Fig. 5e);

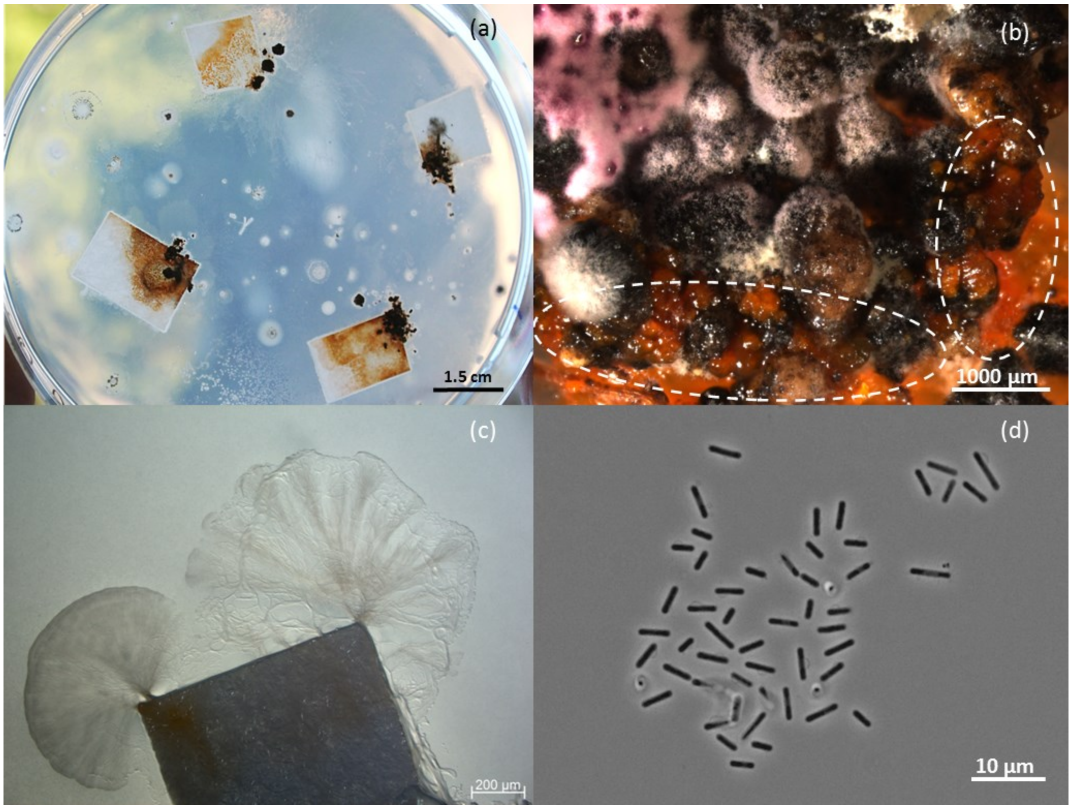

Figure 1. Recognition of cellulose-degrading myxobacteria. (a) Filter-paper baiting method inoculated with soil sample on MS21 mineral salts agar. In the presence of cellulose-degrading myxobacteria, paper usually became yellowish, reddish-orange, brownish, and black color due to the development of swarm and fruiting bodies. (b) The appearance of reddish-orange fruiting bodies (encircled) on filter paper and around the soil inoculum. It is also noticeable that this *Sorangium* strain is growing along with Actinomycetes-like colonies (pink and white powdery growth) on the soil sample. (c) Axenic culture of *Sorangium* showing the swarm stage coming out from the corners of filter paper. (d) Phase-dark and slender rods vegetative cells from the swarming colony.

this is in contrast to the more common strains showing thin swarms with plicate veins on agar (Fig. 5f).

Currently, *Sorangium* is classified into 8 species - *S. cellulosum, S. ambruticinum, S. arenae, S. bulgaricum, S. dawidii, S. kenyense, S. orientale, S. reichenbachii* (Mohr *et al.,* 2019), but with no clear species distinction based on fruiting body, swarm morphology and phenotypic cellulose-degrading pattern. It would be interesting to determine whether the seven new added species differ in color, size, arrangement, and pattern of sporangiole using common lean media including VY/2 agar and mineral salt agar supplemented with filter papers that allow the development of those growth stages. It would also be interesting to compare among those species the swarms and degradation characteristics toward agar and cellulose.

Unlike *Sorangium, Byssovorax* rarely produces fruiting bodies (Reichenbach *et al.,* 2006), but could be recognized by an intense bloody red swarm on filter paper and agar (Fig. 3a-3b). None of the *Sorangium* species has ever been described to have this characteristic. *Byssovorax* has a slimy and soft swarm that makes the filter paper fiber thinner and fragmented after weeks of incubation (Fig. 3c-3d). The fruiting bodies it formed are much bigger and fewer compared with *Sorangium*.

The multi-locus gene and genome comparisons are becoming popular and helpful in describing new bacterial taxa; however, we believe that phenotypic characterizations are as well important, especially in myxobacteria with unique morphology features.

Potential for discovery of novel cellulose-degrading myxobacteria

There is no doubt that new species of *Sorangium* and *Byssovorax* or other cellulose-degrading myxobacteria can be found in the future. Based on BLAST searches, several uncultured bacteria appear related to cellulose-degrading myxobacteria (Table 1). Most of these clones were derived from soils commonly associated with vegetations (Ceja-Navarro *et*

Table 1. Clones derived from uncultured bacteria that are phylogenetically related with *Sorangium* and *Byssovorax* based on 16S rRNA gene sequence. The sequences were found by BLAST searches using the GenBank accession numbers. Clones No. 1-4, neighbor with *Byssovorax;* Clones No. 6-11, neighbor with *Sorangium;* Clone 5 neighbors to both cellulose-degrading genera.

No.	Clone	GenBank Accession No.	Source	Country	Reference
1	S137	DQ083111	soil	not specified	Unpublished
2	AKYG1042	AY921777	farm soil adjacent to a silage storage bunker	Minnesota, USA	Tringe *et al.,* 2005
3	KGB200711-089	EU881272	serious disturbance field soil	Guangxi, China	Unpublished
4	ncd2523b10c1	JF217095	human skin	Washington, USA	Kong *et al.,* 2012
5	DEN_SIP_103	KF533828	full-scale EBPR activated sludge plant	Denmark	Unpublished
6	B8-157	KF494619	permafrost soil	Qinghai-Tibet Plateau, China	Unpublished
7	Plot21-H07	EU193074	agricultural soil	Mexico	Ceja-Navarro *et al.,* 2010
8	Ip 167	KC331385	apple orchard	Loess Plateau, China	Chen *et al.,* 2014
9	p25i20ok	FJ479176	undisturbed tall grass prairie, top 5cm	Kessler Farm, Oklahoma, USA	Youssef *et al.,* 2009
10	FFCH2381	EU134329	soil from an undisturbed mixed grass prairie preserve	Kessler Farm, Oklahoma, USA	Elshahed *et al.,* 2008
11	1112842459896	HQ119466	loamy sand of *Eucalyptus* forest	La Jolla, California, USA	Williamson *et al.,* 2011

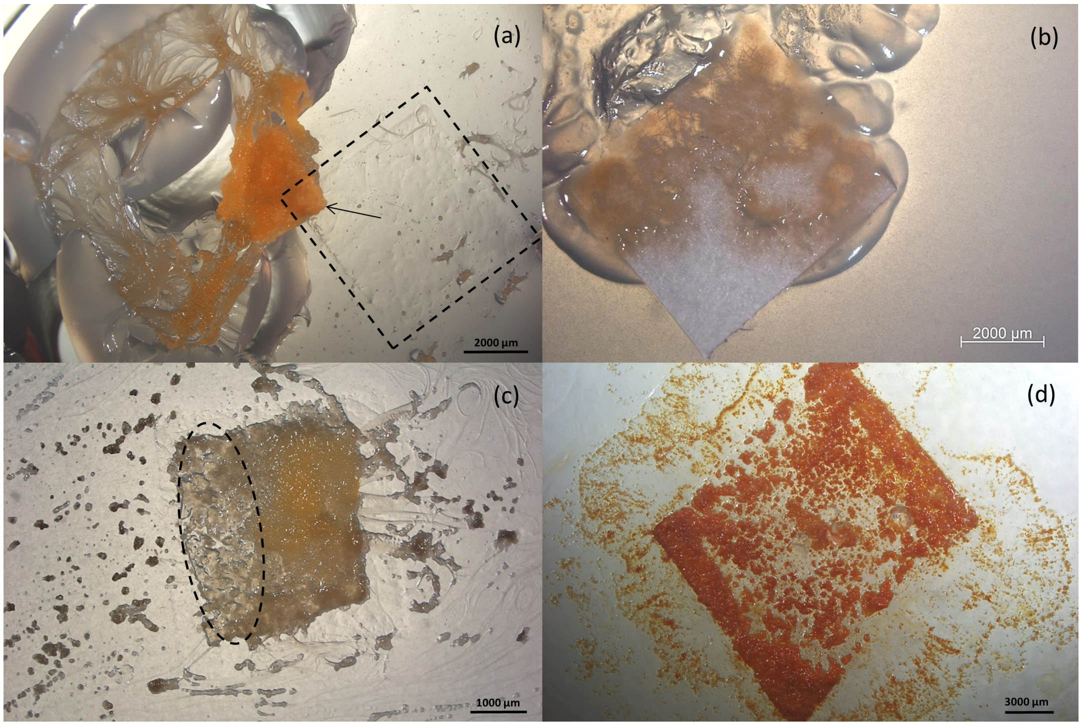

Figure 2. Patterns of cellulose degradation in *Sorangium*. (a) Filter paper is retracted from the agar and crumpled by a stringy and tough swarm. The dotted line enclosed the imprint area of retracted paper (arrow) on agar. Remarkable cuts and breaks in agar can also be recognized in this culture. (b) Strain GT-47^T cellulose degradation along the edges and towards the center of the paper. (c) Filter paper becomes soft, slimy and loosing of the filter fibers (encircled). Yellow cell aggregations are also formed on the paper while brownish-black fruiting bodies can be seen around the paper. (d) Complete cellulose degradation with only fruiting bodies remained to form the shape of a filter paper. Reddish-orange fruiting bodies are also formed closed to the paper and on the agar.

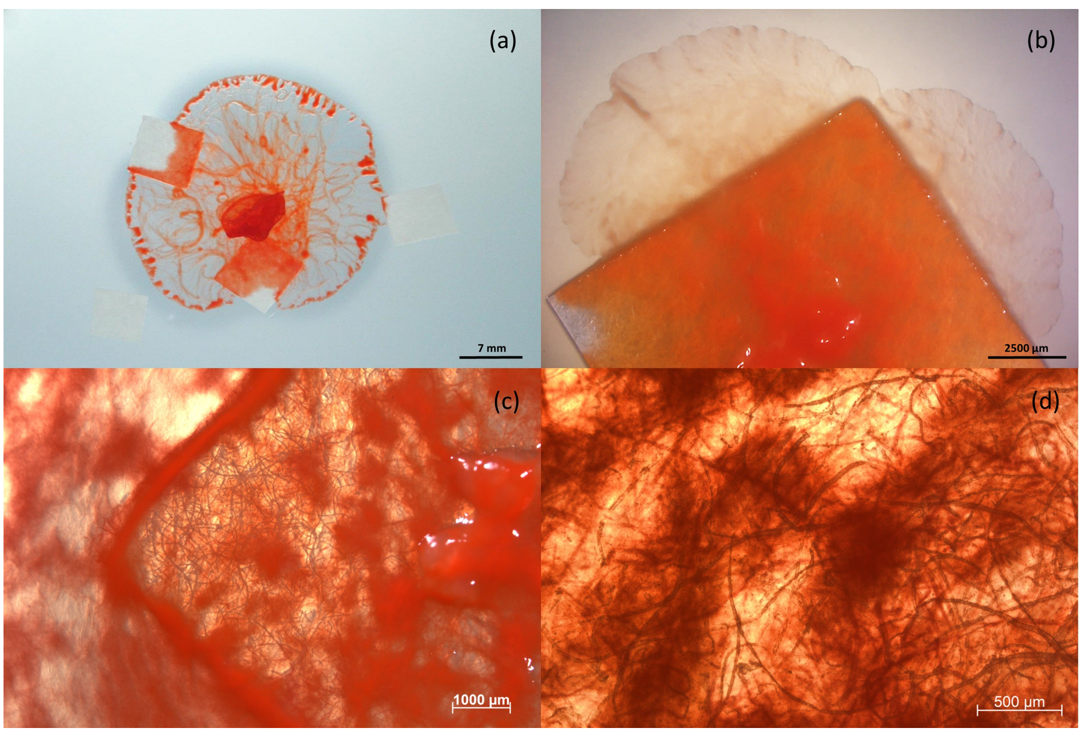

Figure 3. Growth of *Byssovorax cruenta* on VY/2 agar supplemented with maltose. (a) Intense bloody red colony swarming veins on agar and filter paper. (b) Swarms coming out from the edges of a paper. (c) Degradation of cellulose marked by thinning of the paper and showing ripped cellulose fibers. (d) Magnified view of the filter paper showing the fragmentations of the cellulose strands as an implication of degradations.

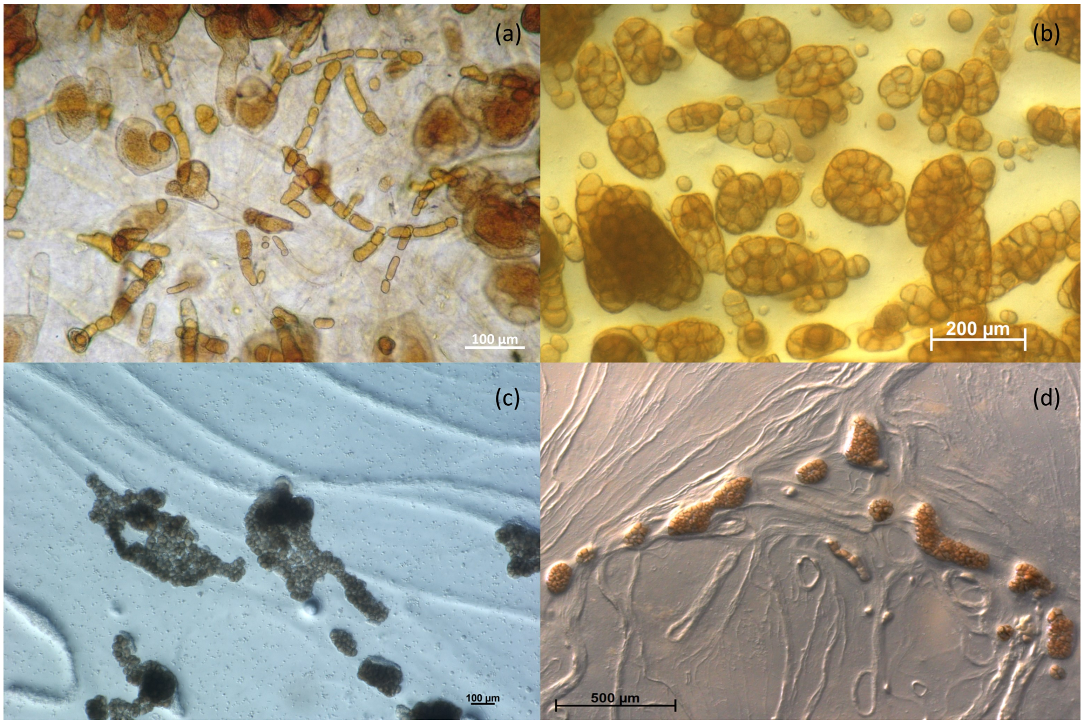

Figure 4. Fruiting body variations in some *Sorangium* strains. (a) Sporangioles appearing mostly as chains in decomposed filter-paper strands. (b) Fruiting bodies compactly arranged as clusters in a completely decomposed filter paper. (c) Black and tiny fruiting bodies on an agar. (d) Mounds of compactly arranged brownish fruiting bodies along with the veiny swarm.

al., 2010; Chen *et al.,* 2014; Elshahed *et al.,* 2008; Williamson *et al.,* 2011; Youssef *et al.,* 2009). This is not surprising since soil is the known habitat for *Sorangium* and *Byssovorax*. Surprisingly, clone ncd2523b10c1 (GenBank Accession number: JF217095) from a human skin associated with atopic dermatitis (Kong *et al.,* 2012) turned out to be 98.6% similar in the 16S rRNA gene sequence with *B. cruenta* By c2^{T} and phylogenetically positioned within the *Byssovorax* clade (Fig. 6). Whether the uncultured bacterial clone ncd2523b10c1 represents just a random occurrence on the skin, a contaminant or even a potential agent to cause disease in humans needs further investigations once this bacterium becomes cultivable under laboratory conditions. So far, none of the isolated and validly described myxobacteria is known to cause any disease in humans.

An indication that cellulose-degrading myxobacteria exist in the extreme environment was based on the metagenomic sequence (clone B8-157, GenBank Accession number KF494619) amplified from a Tibetan Plateau permafrost soil. Based on the 16S rRNA gene phylogenetic analysis, clone B8-157 was determined clustered within *Sorangium* clade and appeared closely related (97.6% similarity) with *S. orientalis* Soce GT47^{T} and *S. arenae* Soce 1078^{T}. Since clone B8-157 occupied a novel branch and showed a 2.4% difference in the 16S rRNA gene sequence, it appears to be a representative of a novel *Sorangium* species after its successful cultivation in the future.

So far, we have found 10 16S rRNA gene sequences (Table 1), all from uncultured bacteria that appear to be related (96.9% - 98.8% similarity) with *Sorangium* and *Byssovorax* after molecular phylogenetic analysis (Fig. 6). Clones 1-4 (GenBank accession numbers: DQ83111, AY921777, EU881272, JF217095) show 97-98.8 % similarity with *Byssovorax* while clones 6-11 (GenBank accession numbers: KF494619, EU193074, KC331385, FJ479176, EU134329, HQ119466) exhibit 96.9% - 98.6% similarity with *Sorangium*. On the other hand, clone 5 (DEN_SIP_103, GenBank accession number: KF533828) from an activated sludge plant in Denmark shows lower similarity (95.2%-95.9%) with *Sorangium* and *Byssovorax* type strains and appears positioned in between these two genera. Clone DEN_SIP_103 possibly represents a novel cellulose-degrading genus in *Polyangiaceae*. By digging on the exact sourced samples as described in the metagenomic studies or by looking at the same type of samples, these uncultured myxobacteria may successfully be brought to cultivation someday. Exploration of various samples obtained from different environments may unexpectedly yield new species of *Sorangium* and *Byssovorax* or related cellulose-degrading taxa

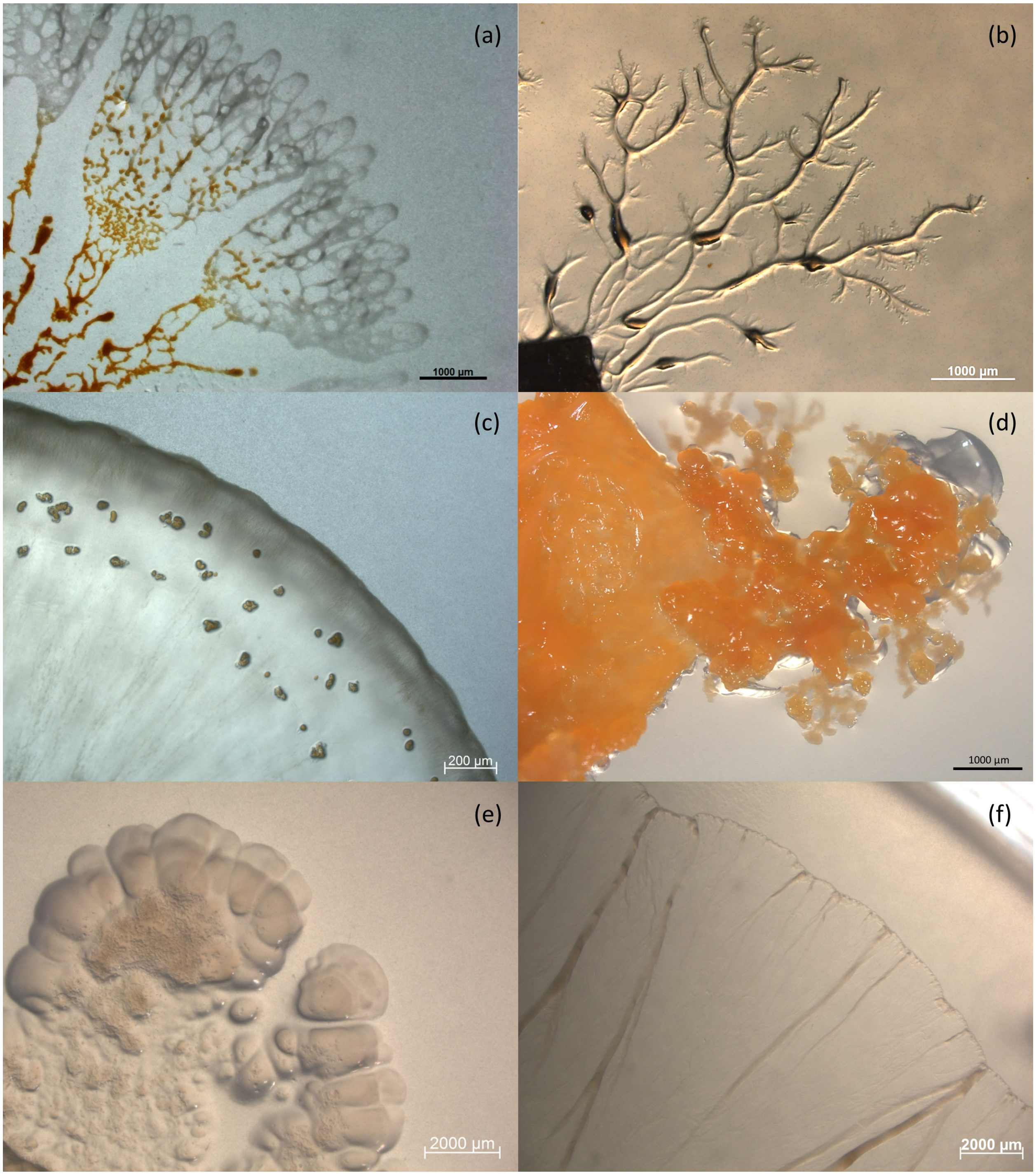

Figure 5. *Sorangium* swarming colony patterns in VY/2 medium baited with filter paper. (a) Net-like veins produced in agar and formation of fruiting bodies behind. (b) Long and branched veins appearing like plant roots on an agar. (c) Fan- or curtain-shaped colony in agar with the partial clearing of the yeast cells and formation of fruiting bodies towards the swarm edge. This reminds similarity with some members in *Polyangiaceae*. (d) Tough and extremely slow-spreading swarm colony causing cuts and ripping of the agar. (e) An unusual colony that grows deep into the agar and creating pits that show similarity with genus *Nannocystis*. (f) Fast-spreading colony on agar marked by swarm plications and long veins.

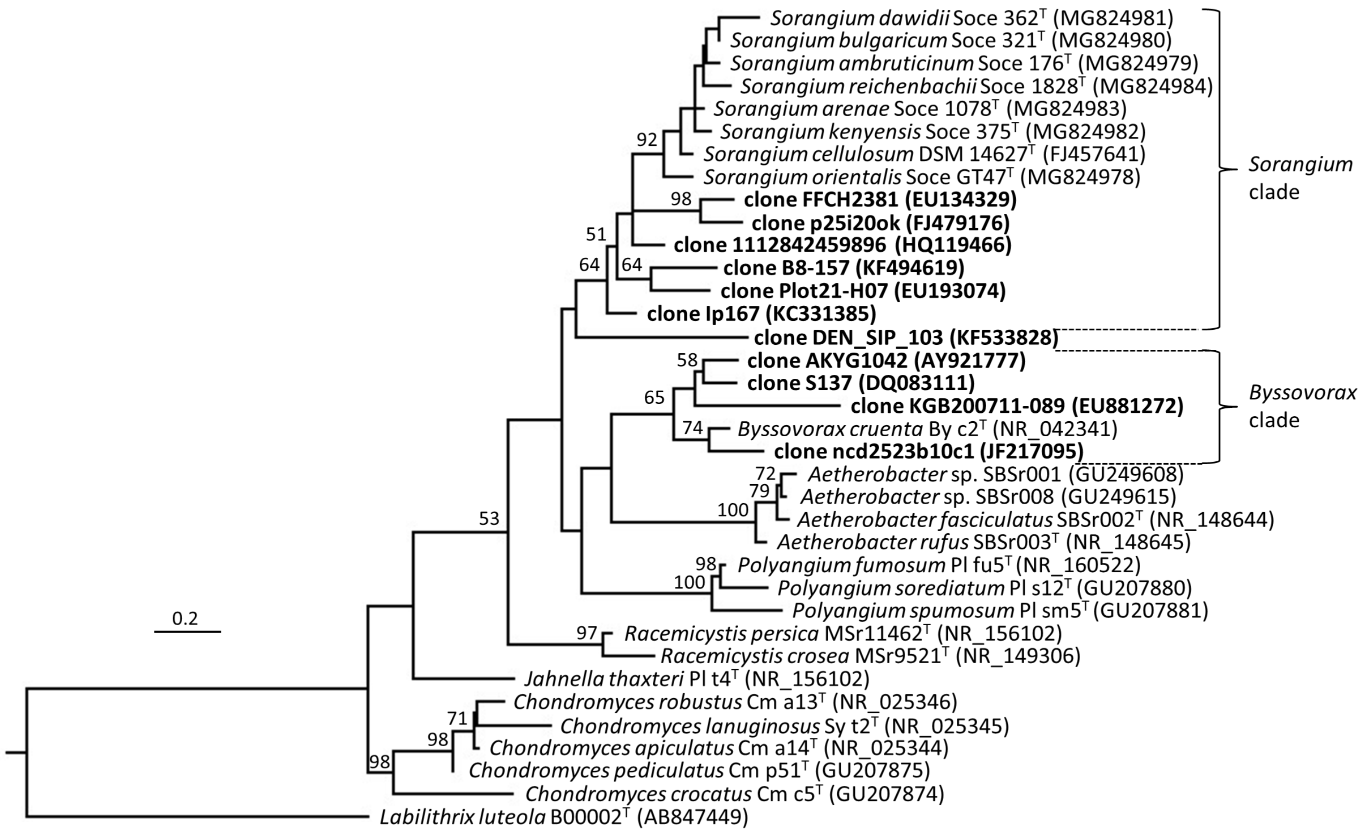

Figure 6. Maximum-likelihood phylogenetic tree inferred from 16S rRNA gene sequence showing the cellulose-degrading myxobacterial clades in *Sorangium* and *Byssovorax*. The tree also shows the position of clone-derived sequences from uncultured bacteria (boldface). *Labilithrix luteola* B00002^{T} was used as an outgroup to root the tree. Bootstrap values greater than 50% are shown at the nodes based on 1000 replications. The GenBank accession numbers are indicated in parenthesis. The bar represents 20 nucleotide substitutions per 1000 sites.

in the future.

The uncultured myxobacteria in *Sorangium* and *Byssovorax* clades are likely sharing distinct features with these genera. Since they are also located in *Polyangiaceae* family, phenotypically they are expected to share the same cigar-shaped vegetative cell-type capable of agar degradation. They are also presumed to produce swarms (as bands or pseudoplasmodia) that can penetrate the agar and at the same time capable of degrading cellulose. In addition, these uncultured strains may be hypothesized to require longer incubation time, thus can be recognized by their colonies and fruiting bodies after weeks or months of cultivations. All these characteristics may be considered in the isolation of these new potential species in the *Sorangium* and *Byssovorax* clades.

Concluding remarks and future research direction

This phylogenetic study based on metagenome data shows that there are at least several potentially new species of cellulose -degrading myxobacteria in *Sorangium* and *Byssovorax* clades. Candidate new genera related to *Sorangium* and *Byssovorax* can also be discovered and isolated in the future. Since they are currently inaccessible and only known to-date as "uncultured bacteria", we speculate they share phenotypic characteristics with currently known cellulolytic myxobacteria, and we hope that this will serve as a guide in bringing them to laboratory cultivations. Some motivations for isolating these groups of "uncultured cellulolytic myxobacteria" apart from expanding the taxonomic diversity are probably due to their medical and industrial applications to discover new anti-infectives and anti-cancer drugs.

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